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A STUDY OF SOME FACTORS IN THE CHEMICAL STIMULATION OF THE GROWTH OF ASPERGILLUS NIGER

Robert Aaron Steinberg

The distinction between "nutrients" and "stimulants" has been the subject of intensive study since synthetic culture media of known composition have come into use. It was early observed that substances which were considered to be by no means essential constituents of protoplasm might still produce a marked increase of growth. The phenomena of so-called "chemical stimulation" due to the addition to a nutrient solution of substances not regarded as essential for growth are apparently of general occurrence. Such phenomena have been described for bacteria (16), fungi (57), algae (49), and the higher plants (25, 7, 33). These studies for the most part had for their aim to ascertain the effect of the substance in question on the growth and on respiration. I have reported elsewhere (64) on evidence that substances may be dissolved in sufficient quantity from the glass of the cultural flasks to cause a partial or even a maximum acceleration of growth. I have also presented evidence that the amount of growth as well as the concentration of "stimulant" necessary for its production varies with the strain of *Aspergillus niger* used (65). I shall report here on further experiments testing the significance of the degree of acidity of the culture medium and the effect of varying its iron or zinc content singly or together. These results are obtained by the use of a method of purifying the culture medium not hitherto used in such physiological experiments. Lastly I have attempted to correlate the facts in the literature of "chemical stimulation" with some of the newer data on the problems of nutrition and growth obtained by other methods.

The studies of Pasteur (51) on the alcoholic fermentation of yeast led to extensive investigations of the food requirements of the fungi. It was determined by Pasteur in the course of his investigations that yeast could grow in an aqueous sugar solution containing ammonium tartrate and yeast ash. Beyond ascertaining that the yeast ashes could not be replaced by magnesium phosphate and a few other salts, the matter was dropped. No attempt was made systematically to investigate the elements known by him to be present, from Mitscherlich's (38) analyses, in the yeast ash. The analyses referred to are shown on the following page.

The continuation by Raulin (54), a student of Pasteur, of these studies on the components of the yeast ash in relation to the growth of another fungus, "*Ascophora nigrans*" (*Aspergillus niger*), soon followed. Raulin

Composition of Ash

	Top Yeast	Bottom Yeast
H_3PO_4	41.8%	39.5%
K	39.8	28.3
Na	—	—
$CaHPO_4$	2.3	9.7
$MgHPO_4$	16.8	22.6
SiO_2	tr.	—

substituted various compounds, singly and combined, for the yeast ash in the Pasteur nutrient solution and determined the extent of growth as represented by the dry weight of the tissue formed. The "most useful" substances in the order of their importance were, according to Raulin: ammonium phosphate, potassium carbonate, magnesium carbonate, ammonium sulphate, and manganese carbonate.

In 1869 a continuation of these studies was published by Raulin (55), and almost simultaneously appeared an important contribution by A. Mayer (38).

Raulin had continued his studies on *Aspergillus niger*, arriving at a nutrient solution "essai type" which contained, as he held, all the elements "essential" for the growth of this organism in the proper proportions. The omission of any component of his "essai type" solution resulted, he stated, in a large decrease in growth. I repeat the formula as a matter of historical interest: water 1,500 g., cane sugar 70.00 g., tartaric acid 4.00 g., ammonium nitrate 4.00 g., ammonium phosphate 0.60 g., potassium carbonate 0.60 g., magnesium carbonate 0.40 g., ammonium sulphate 0.25 g., zinc sulphate 0.07 g., iron sulphate 0.07 g., potassium silicate 0.07 g.

As a result of his studies on the "normal" development and respiration of a yeast, Mayer (38) had arrived, however, at a much simpler nntrient solution, containing, if we exclude from consideration the water and cane sugar, but four compounds as compared to the nine entering into the composition of the nutrient solution as worked out by Raulin. The elements Mayer considered "essential" are present in this solution in the proportions most favorable for "normal" development and respiration. Moreover, these proportions, Mayer points out, closely approximate those found in the yeast ash. The formula of the Mayer solution is: water 1000.0 g., cane sugar 150.0 g., ammonium nitrate 10.0 g., monopotassium phosphate 5.0 g., magnesium sulphate 2.5 g., calcium phosphate ($CaHPO_4$) 0.5 g.

A comparison of these two formulae with respect to the elements present shows a substantial agreement. Both solutions contain hydrogen, oxygen, carbon, nitrogen, phosphorus, sulphur, potassium, and magnesium. Raulin included in addition zinc, iron and silicon; Mayer, calcium only.

With respect to the necessity of silicon the evidence is as yet indecisive. Calcium has, however, been shown by Benecke (3) not to be an "essential" element in the nutrition of fungi.

The necessity of iron for the fungus has been a much disputed question. That iron is an "essential" participant in the metabolism of the fungus is the opinion of Molisch (45). Benecke's (3) experimental results, as he himself points out, are inconclusive. More recently Currie (11) has concluded that the exclusion of iron from the nutrient solution is without effect on the growth and reproduction of *A. niger.*

Von Nägeli (46) concluded that certain substitutions are possible. Thus, he thought, rubidium or caesium could be used in place of potassium; and calcium, barium, or strontium in place of magnesium. That Nägeli was in error has been shown by Benecke (3), inasmuch as in the entire absence of either potassium or magnesium he could obtain no growth. Benecke found, however, that partial replacement was possible: greater growth took place but the cultures did not fruit. This non-sporulation is, at least in the case of rubidium and caesium, not to be confused with an analogous phenomenon accompanying "stimulation" (see below) however, but is simply due according to Sauton (61) to insufficient potassium and not to a "sterilizing action" of the caesium and rubidium.

The nutrient solution utilized by Pfeffer (52), which is quite similar to that worked out by Mayer, is being increasingly made use of in nutritional studies. It is perhaps the simplest of the nutrient solutions in use.

PFEFFER SOLUTION

Water	1000.0 g.
Cane sugar	50.0 g.
Ammonium nitrate	10.0 g.
Monopotassium phosphate	5.0 g.
Magnesium sulphate	2.5 g.
Ferrous sulphate	trace

As respects the action of the components of the nutrient solution, it was early partially recognized by v. Nägeli (46, 47) that the action of a compound on the growth of an organism depends on and increases with its concentration, and that the presence of other compounds can cause a modification of this action. Each compound, according to him, exercises a "specific action" which is apart from and without reference to its nutritive value. Every compound has an "optimum" concentration for growth; and a "schädliche" (toxic) concentration. The latter is higher in value than the "optimum." Both the "optimum" and the "toxic" concentration vary with the compound and with the particular organism used. In other words, the "optimum" concentration of any compound in a nutrient solution varies with the composition and concentration of each of the other compounds present and with the organism.

Much evidence has accumulated showing the specific effect of "poisons," "stimulants," etc., on the growth of organisms. That in sufficiently high dilution many "poisons" are not harmful, but may cause an acceleration of the life activities of the organism was apparently known to Braconnot (6) in 1845. Schulz's (62) studies on yeast demonstrated that the presence of such substances may result in increased respiration. The rôle of zinc in the nutrition of *A. niger* and other fungi was apparently first considered as an analogous phenomenon by Pfeffer (52). Richards (57) on the basis of a broad comparative study concluded that the action of zinc is shared by many other substances, such as iron, lithium, cobalt, nickel, fluorides, etc., and even alkaloids; and that zinc therefore cannot be regarded as more "essential" than many other elements.

Since the initial research of Raulin (55), by which was indicated the increased yield resultant upon the addition of zinc to cultures of *Aspergillus niger*, various other (approximately seventeen) ions have been shown to act in a similar manner. "The increased growth," to quote Pfeffer (53, p. 146), "appears to be due to a general power of reacting against injurious influences possessed by living organisms, for similar results are produced by ether, alkaloids, etc., not only upon growth, but also upon respiration and fermentative activity." Further: "A very strong poison produces its optimal stimulating effect when extremely dilute and growth may be retarded by doses above the optimum, while substances which act as poisons only when highly concentrated produce no perceptible result at all."

The acceleration of growth of *Aspergillus niger* may appear in the presence of very diverse carbon (57, 56) and nitrogen (57, 22) sources. The increase in respiration has been studied by Schulz (62) with yeasts; and Richards (58), Ono (49), and others have pointed out that the "economic coefficient" $\left(\frac{\text{dry-weight formed}}{\text{100 g. sugar used}}\right)$ is increased in the presence of zinc. The total acidity of the culture solution, it has been found by Currie (11), increases more rapidly in the "stimulated" cultures than in the controls. This condition is reversed after some time.

The increased growth involves (Fred, 16) an increased number of cell divisions.

We may next note the evidence from the literature for increased growth as dependent on the acidity of the culture medium. Attention has already been drawn by Mathews (37) to the fact that the hydrolytic dissociation of the salts of the heavy metals is an important factor in their action, as shown by their precipitating power. Similarly, Mines (44) has pointed out that as a result of hydrolytic dissociation the action of an electrolyte may be either increased or decreased. Of interest in this connection are certain experiments by Pantanelli (50), who, in his studies on the selective absorption of ions from single salt solutions by *Cystosira amentacca*, found that in practically every case the H-ion concentration of the solution increased considerably after an immersion of two hours. The action of the salts of the heavy metals, which upon hydrolytic dissociation give an increased acidity of the solution (see for example Denham, 13), will probably be found to have been over- or underestimated on this account.

Evidence for the action of the hydrogen ion on growth and reproduction has been many times presented, and a direct correlation of this effect with that of the ions exercising a "stimulative effect" is perhaps possible.

Raulin (55, p. 162) pointed out quite clearly that a definite optimum acidity or alkalinity exists for the growth of each organism, and further that the optimum reaction varies with the organism. The "Mucédinees" and the "Mycodermes," he stated, prefer an acid medium; the "végétaux-ferments" a neutral; and the "Infusoires" an alkaline medium. Raulin himself utilized tartaric acid in his nutrient solution in order to favor the growth of *Aspergillus niger* and to exclude "Infusoires." With high concentrations he reports a retardation or suppression of growth and spore formation.

It has come to be a commonplace that fungi as a rule prefer an acid, and bacteria an alkaline, medium. This is the basis of the conception of many of the early students of plant diseases that only fungi and not bacteria could cause plant diseases; the bacterial diseases being confined to animal organisms whose protoplasm is alkaline (see Smith, 63). Similar statements with regard to the acidity requirements of fungi and bacteria have been repeated with great frequency, as for example by v. Nägeli (46, p. 63), Pfeffer (53, p. 384), and Jost (23, p. 205), without as a rule any definite statements as to the actual effects of acidity on the growth of any one organism.

As respects the algae, we may note that Migula (43) observed that very slight increases in acidity cause an acceleration of the growth in length. Cell division, however, seems not to have been accelerated.

For the higher plants as grown in water culture, A. Mayer (39, p. 273), Pfeffer (53, p. 415), Jost (23, p. 92), and even Russell (60, p. 49) in 1917, refer to the effects of acidity only vaguely.

Within comparatively recent times *quantitative* experimental evidence on the relation of acidity to growth has begun to accumulate. The discovery of Kahlenberg and True (24) that the toxicity of acids depends on their ionization (*i.e.*, the resulting H-ion concentration) has been of great importance and significance. We need only consult Michaelis' (41) summary of the literature to realize the fundamental importance of the H-ion concentration for enzymatic action, etc.

Clark (9) states that for a number of acids the growth of *A. niger* varies with the acidity: ". . . ¼ to ⅛ the lethal concentration has a strong stimulating influence on the mycelial development, and tends to suppress or at least retard fructification" (p. 294). Unfortunately no yields are given, and the acidities are calculated from the ionization of the acids in water though added in this case to a sugar-beet decoction. Fred and Loomis (17) have studied the influence of the H-ion concentration of the medium on the reproduction of alfalfa bacteria. The optimum acidity they note corresponds to $p_H = 7.2$; at $p_H = 3.10$ no growth occurs. [$p_H = -\log_{10}$

(H^+), *i.e.*, the negative logarithm of the hydrogen-ion concentration in gram-ions.] Furthermore, they note the same phenomenon observed by Hoagland (20) with barley; the acidity of the medium is modified as a result of growth, increasing where the initial acidities were too low, decreasing where the initial acidities were too high. Hoagland found $p_H = 5.2$ the optimum acidity for barley plants.

There is also some evidence bearing out the view of Pfeffer (53, p. 487) that a specific limiting acidity is reached as the result of the growth, etc., of certain organisms. Michaelis and Marcora (42) have found it to hold true in the case of *Bacterium coli;* Clark and Lubs (10) with *Aspergillus niger;* and Ayers (2) with many streptococci. This fact is at the basis of the procedure used in the laboratory of the addition of $CaCO_3$ in lactic acid fermentation. As is similarly generally recognized (53) an analogous phenomenon occurs in acetic acid, and in alcoholic, fermentation; and probably also in butyric acid fermentation. It is, we may say, frequently the case that the growth of an organism is limited by the attainment of a definite concentration of some acid produced by its own metabolism.

Experiments on the relation of the acidity of the Pfeffer nutrient solution to the growth and fructification of *Aspergillus niger* under conditions known to exclude to a high degree the "stimulative" ions, were undertaken in order to obtain cultures directly comparable with zinc cultures.

METHODS

In the following experiments the Pfeffer nutrient solution was used exclusively. The compounds employed in the preparation of this solution were Merck's "Reagent" ammonium nitrate and magnesium sulphate; and Baker's "Analysed" potassium phosphate and ferric sulphate; the water was redistilled through glass (the first and last quarters of the distillate are rejected). The cane sugar used is that sold under the proprietary name of "Crystal Domino" sugar. The use of other compounds is always noted in the description accompanying the experiments. Tests with *A. niger* showed that the results obtained with "Crystal Domino" sugar in nowise differed from those obtained with Merck's "Highest Purity" cane sugar. In both cases growth proceeded as well with as without the addition of a trace of iron salt; and in the presence of zinc a marked acceleration of growth occurred. The information was obtained on inquiry from the manufacturer that no coloring matter is added to this sugar in the refining process, and that this practice had gone out of use over thirteen years ago.

The flasks used were 150 cc. pyrex Erlenmeyers. They were cleaned by rinsing with concentrated sulphuric acid (Baker's "Special"), tap-water, lastly with distilled water, and inverted to drain dry. It may be noted here that when zinc is present in the glass of the cultural flasks (Jena glass) sufficient amounts may go into solution to cause partial or maximum "stimulation," as I have shown elsewhere (64).

Results

1. The influence of a zinc glass (Jena) on the yield [Reproduced from (64)].

$MgSO_4.7H_2O$ (Kahlb.) Room temperature (18–23° C.). Period of growth ten days.

Jena		Kavalier Bohemian		Pyrex	
No Zinc	10 Mg. Zn/L	No Zinc	10 Mg. Zn/L	No Zinc	10 Mg. Zn/L
0.989 g.	0.980 g.	0.270 g.	0.924 g.	0.319 g.	0.940 g.
0.958	0.940	0.299	0.943	0.248	0.980
0.919	0.988	0.285	0.886	0.306	0.917
0.933	1.005	0.300	0.947	0.252	0.952
0.953	1.022	0.361	1.017	0.309	0.997
0.950	0.987	0.301	0.943	0.287	0.957

2. The influence of a zinc glass (Jena) on the comparative yield of two strains of *A. niger* (W and Y).

$MgSO_4.7H_2O$ (Kahlb.)

Strain W, Transfer 1			Strain Y, Transfer 1		
Pyrex		Jena	Pyrex		Jena
No Zinc	0.01 Mg. Zn/L	No Zinc	No Zinc	0.01 Mg. Zn/L	No Zinc
0.302 g.	0.602 g.	0.805 g.	0.516 g.	0.806 g.	0.763 g.
0.265	0.578	0.741	0.535	0.830	0.747
0.339	0.562	0.810	0.546	0.843	0.785
0.330	0.528	0.786	0.409	0.694	0.750
0.335	0.554	0.811	0.393	0.732	0.787
0.317	0.565	0.791	0.480	0.781	0.766

In Jena glass, it will be noted (experiments 1, 2) that the maximum growth occurs whether zinc is added or not.

The following experiment reproduced from a former publication (64) will also show that the method used in cleaning the flasks is entirely satisfactory. The flasks used for the cultures in the second column were previous to this experiment used three consecutive times for cultures containing 10 Zn/L and were cleaned as usual.

3. On the efficiency of the method for cleaning the cultural flasks.

$MgSO_4.7H_2O$ (Kahlb). Pyrex flasks.

Flasks Reserved for Zinc-free Cultures	Flasks Reserved for Zinc Cultures
* 0.924 g.	0.283 g.
0.336	0.265
0.321	0.313
0.328	0.341
0.355	0.352
0.335	0.311

* An accidental zinc culture. In some cases due to the accidental introduction of zinc, in a few cases to the unintentional use of Jena flasks.

The addition of zinc, etc., where indicated was to the entire solution used in the preparation of cultures having the same concentration of this heavy metal, etc., and not to the individual flasks. Exceptions to this rule, however, were frequently made when only two or three duplicate cultures were prepared. Zinc was always added from one or two stock solutions ($ZnSO_4.7H_2O$, Baker's "Analysed"). One contained 2.5 mg. zinc per cubic centimeter (*i.e.*, 11.0 mg. $ZnSO_4.7H_2O$ per cc.); the other 0.025 mg. zinc per cubic centimeter.

The flasks, each containing 50 cc. of nutrient solution, measured in a 50 cc. graduate, were sterilized at 14½ lbs. for 20 minutes.

Inoculations of the flasks were always from bread cultures in jelly glasses having tinned covers. In order to maintain uniform moisture conditions, the bread (a portion of a slice of fresh bread one inch thick) was supported in the center of the glass by means of a horizontally placed microscope slide so that it did not come in contact with the water in the bottom of the glass. In their preparation the water should be omitted until after sterilization (14½ lbs. for 30 minutes). Immediately after sterilization and while still hot, the proper amount (50 cc.) of sterile water (it should be autoclaved at the same time as are the cultures) was added. In this manner a large surface of growth for the organism was obtained under fairly uniform moisture conditions, and the cultures were ready for inoculation even after several months. Enough spores[1] were added from the bread culture by means of a platinum loop to make a visible and apparently almost continuous layer on the surface of the solution in the flask. Immediately after inoculation the flasks were placed in an incubator (with exclusion of light) at 30–31° C. for seven days.

When harvested, the membrane, together with the solution, was thrown on a washed and weighed filter, washed twice with distilled water and dried at 103–105° C. for four days.

The yields, while given to three places, are probably reliable to the second place only.

It will be noted that the experimental conditions are not entirely uniform. Particularly is this the case with the *A. niger* cultures used. The *A. niger* culture used in these experiments was obtained originally from the "Internationalstelle für Pilz-Kulturen," Amsterdam. Later the two one-spore cultures referred to above as W and Y were isolated from this culture in the manner described in an earlier paper (65).

The two strains thus isolated were kept on agar slants (1 % each of peptone, sucrose, and agar), in the incubator at 30–31° C. Sub-cultures (on bread) for the inoculation of the flasks were also kept in the incubator at 30–31° C. The stock cultures were carried in duplicate, transfers being at irregular intervals. A duplicate stock culture once having been used, for the preparation either of new stock cultures or of a bread culture, was placed aside as unfit for further use. The practise was also followed of preserving a single duplicate stock culture, unused, from each transfer. The numbers given with the designation of the strain refer to the total number of transfers since the original isolation of the strains.

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[1] Approximately four milligrams dry weight.

I present here summaries of three of the 18 experiments presented in the

4. The effect of adding zinc to the culture medium (strain W). Strain W, 6. Each value the average of five duplicate cultures.

Mg. Zn/L	Yield	Sporulation	Mg. Zn/L	Yield	Sporulation
—	0.136 g.	Excellent.	5.0	0.963 g.	Sterile.
0.01	0.287	"	10.0	0.906	"
0.1	0.851	Good.	15.0	0.902	"
0.5	0.843	Moderate.	20.0	0.901	"
1.0	0.915	"	25.0	0.945	"

5. The effect of adding zinc to the culture medium (strain Y). Strain Y, 6. Each value the average of five duplicate cultures.

Mg. Zn/L	Yield	Sporulation	Mg. Zn/L	Yield	Sporulation
—	0.205 g.	Excellent.	5.0	0.757 g.	Good.
0.01	0.283	"	10.0	0.771	"
0.1	0.823	"	15.0	0.746	Fair.
0.5	0.826	Good.	20.0	0.725	"
1.0	0.859	"	25.0	0.754	"

6. The effect of adding zinc to the culture medium (Strains W and Y).

Each value the average of two duplicate cultures, except the controls which are the average of five duplicate cultures.

Strain W, 10

Mg. Zn/L	Yield	Sporulation	Methyl Violet
—	0.177 g.	Excellent.	Blue.
0.01	0.287	"	"
0.02	0.381	"	"
0.03	0.475	"	"
0.04	0.523	"	Green-blue.
0.05	0.534	"	"
0.06	0.688	"	Blue-green.
0.07	0.697	"	"
0.08	0.758	"	Green.
0.09	0.761	"	"
0.10	0.800	"	"

Strain Y, 10

Mg. Zn/L	Yield	Sporulation	Methyl Violet
—	0.222 g.	Excellent.	Blue.
0.01	0.440	"	Green-blue.
0.02	0.501	"	"
0.03	0.563	"	"
0.04	0.642	"	Blue-green.
0.05	0.707	Fair.	Green.
0.06	0.793	"	"
0.07	0.753	"	"
0.08	0.734	Practically sterile.	"
0.09	0.770	" "	"
0.10	0.799	" "	"

paper referred to above (65). We may note that both the zinc optimum and the maximum yield of the two strains differed (experiments 4, 5, and 6).

The above noted cultures indicate also the nature of the results obtained by the addition of zinc sulphate to the Pfeffer nutrient solution. We note that with increasing concentrations of zinc the yield increased and spore formation decreased. The maximum acceleration of growth occurred in the case of both strains in a zinc concentration of 0.1 mg. Zn/L or less, higher concentrations of zinc still resulting in the maximum growth or in a slight decrease. The optimum concentration of zinc for growth as well as the maximum growth obtained is greater for the W than for the Y strain.

Two explanations have been suggested for the method of action of these ions on growth. Javillier (22) is inclined to believe that the increased growth on addition of zinc is due essentially to the increased elaboration of an enzyme—sucrase. The evidence offered does not, however, settle the question as to whether the increased production of sucrase by the organism is the result or the cause of the accelerated growth.

Further there is the suggestion of Loeb (35) that the phenomenon depends on the balancing of the solution. Of this, however, I could find no evidence in my studies. Variation of the concentration and proportions of the constituents of the Pfeffer solution (except iron sulphate) in no case led to the exhibition of phenomena at all comparable to those accompanying the action of zinc. That is, no indication could be obtained that either ammonium, potassium, hydrogen, or magnesium ions were present in toxic concentration. Experiments 7 to 16 inclusive will make this clear.

The Pfeffer nutrient solution contains NH_4NO_3, KH_2PO_4, and $MgSO_4.7H_2O$ in amounts of 10 : 5 : 2.5 grams respectively per liter. The compositions of the following solutions have been indicated in the same manner.

7. The effect of a decrease in concentration of the magnesium sulphate.

4:2:1*	4 : 2 : 0.2	4 : 2 : 0.04	4 : 2 : 0.008
0.139 g.	0.127 g.	0.093 g.	0.038 g.
0.142	0.118	0.074	0.035
0.151	0.110	0.083	0.035
0.131	0.113	0.092	0.032
0.169	0.115	0.091	0.036
0.146	0.117	0.087	0.035

NH_4NO_3		KH_2PO_4		$MgSO_4 \cdot 7H_2O$	Sporulation
4	:	2	:	1	Very good.
4	:	2	:	0.2	Good.
4	:	2	:	0.04	Fair.
4	:	2	:	0.008	Very sparse, very delicate mycelium.

* $MgSO_4.7H_2O$ (Kahlbaum).

8. The effect of a decrease in concentration of one or more of the salts of the Pfeffer solution.

10 : 5 : 2.5*	10 : 5 : 1	10 : 2 : 2.5	10 : 2 : 1
† 0.917 g. 0.294 0.303 0.261 0.342	0.335 g. 0.311 0.397 0.367 0.415	0.343 g. 0.255 0.244 0.271 0.304	0.297 g. 0.276 0.301 0.300 0.321
0.300	0.365	0.284	0.299
4 : 5 : 2.5	4 : 5 : 1	4 : 2 : 2.5	4 : 2 : 1
0.254 g. 0.207 0.254 0.223 0.152	0.238 g. 0.280 0.229 0.243 0.336	0.284 g. 0.164 0.162 0.163 0.178	† 0.796 g. 0.179 0.176 0.187 0.189
0.218	0.265	0.190	0.183

* $MgSO_4.7H_2O$ (Kahlbaum).

† Accidental zinc culture.

All fruited in two days.

9. The effect of a decrease in concentration of the monopotassium phosphate.

4 : 2 : 0.01‡	4 : 0.4 : 0.01	4 : 0.08 : 0.01	4 : 0.016 : 0.01
0.128 g. 0.085 0.106 0.245 0.107	0.104 g. 0.121 0.138 0.089 0.108	0.110 g. 0.158 0.117 0.137 0.119	0.097 g. 0.078 0.082 0.077 0.088
0.134	0.112	0.128	0.084

‡ $MgSO_4.7H_2O$ (Kahlbaum).

In these experiments we note that only a decrease in the yield follows a decrease in concentration of one or more of the inorganic constituents of the Pfeffer solution, and we may conclude therefore that none of them are present in toxic or in greater than optimum concentration.

The effects of increasing the concentration of the electrolytes of the Pfeffer nutrient solution as well as of a decrease are shown in experiments 10–16 inclusive.

10. The effect of a variation in concentration of the monopotassium phosphate in the Pfeffer solution.

10 : 25 : 2.5 §	10 : 10 : 2.5	10 : 0.1 : 2.5	10 : 0.01 : 2.5
0.496 g. 0.389 0.349 0.459 0.556	0.335 g. 0.327 0.355 0.334 0.266	0.196 g. 0.182 0.136 0.159 0.169	0.048 g. 0.048 0.053 0.058 0.065
0.450	0.323	0.168	0.054

§ $MgSO_4.7H_2O$ (Kahlbaum).

In all except 10 : 0.01 : 2.5 (NH_4NO_3 : KH_2PO_4 : $MgSO_4.7H_2O$), good sporulation was obtained; in the latter slightly less sporulation and no membrane, but the solution was full of submerged hyphae, apparently in a solid mass.

11. The effect of a variation in concentration of the ammonium nitrate in the Pfeffer solution.

25 : 5 : 2.5*	15 : 5 : 2.5	0.1 : 5 : 2.5	0.01 : 5 : 2.5
0.430 g.	0.409 g.	0.070 g.	0.023 g.
0.346	0.372	0.058	0.022
0.403	0.329	0.060	0.029
0.368	0.324	0.057	0.030
0.331	0.363	0.061	0.042
0.376	0.359	0.061	0.029

NH_4NO_3	KH_2PO_4	$MgSO_4.7H_2O$	Sporulation
25	5	2.5	Good.
15	5	2.5	"
0.1	5	2.5	Sparse, solid mass submerged hyphae and membrane.
0.01	5	2.5	Very sparse, solid mass submerged hyphae and membrane.

* $MgSO_4.7H_2O$ (Kahlbaum).

12. The effect of a variation in concentration of the magnesium sulphate in the Pfeffer solution.

10 : 5 : 25	10 : 5 : 10	10 : 5 : 0.1	10 : 5 : 0.01
0.552 g.	0.360 g.	0.156 g.	0.087 g.
0.450	0.353	0.127	0.087
0.487	0.387	0.178	0.112
0.540	0.372	0.182	0.121
0.488	0.371	0.284	0.118
0.503	0.369	0.185	0.105

Sporulation in all except 10 : 5 : 0.01, good; in latter, fair.

In experiments 10, 11, and 12 we find that if we vary the concentration of ammonium nitrate, of monopotassium phosphate, or of magnesium sulphate in the Pfeffer solution, the others being held constant, the effect of a decreased concentration is a decreased yield, that of an increased concentration an increased yield.

13. The effect of an increase in concentration of the monopotassium phosphate in the Pfeffer solution.

Control	Strain W, 5 KH_2PO_4	Yield
0.133 g.	10 : 29 : 2.5	0.153 g.
0.124	10 : 45 : 2.5	0.246
0.146	10 : 65 : 2.5	0.190
0.140	10 : 85 : 2.5	0.381
0.121	10 : 105 : 2.5	0.369
0.133		

14. The effect of increasing the concentration of the magnesium sulphate in the Pfeffer solution.

$MgSO_4.7H_2O$ (Kahlbaum).

Strain W, 1		Strain Y, 1	
Control	0.01 Mg. Zn/L	Control	0.01 Mg. Zn/L
0.302 g.	0.602 g.	0.516 g.	0.806 g.
0.265	0.578	0.535	0.830
0.339	0.562	0.546	0.843
0.330	0.528	0.409	0.694
0.335	0.554	0.393	0.732
0.317	0.565	0.480	0.781

Strain W, 1

10 : 5 : 22.5	10 : 5 : 42.5	10 : 5 : 62.5	10 : 5 : 82.5	10 : 5 : 102.5
0.530 g.	0.586 g.	0.669 g.	0.621 g.	0.810 g.
0.372	0.448	0.638	0.730	0.907
0.451	0.517	0.654	0.676	0.859

Strain Y, 1

10 : 5 : 22.5	10 : 5 : 42.5	10 : 5 : 62.5	10 : 5 : 82.5	10 : 5 : 102.5
0.600 g.	0.663 g.	0.817 g.	0.746 g.	0.980 g.
0.487	0.600	0.757	0.775	1.017
0.544	0.632	0.787	0.761	0.999

Retardation of spore formation only in 10 : 5 : 102.5 (NH_4NO_3 : KH_2PO_4 : $MgSO_4.7H_2O$). With increased concentrations of $MgSO_4.7H_2O$ the spores became more and more brown colored.

15. The effect of increasing the concentration of the magnesium sulphate in the Pfeffer solution.

Strain W, 2

10 : 5 : 2.5	10 : 5 : 77.5	10 : 5 : 83.75	10 : 5 : 90	10 : 5 : 96.25
0.224 g.	0.613 g.	0.646 g.	0.695 g.	0.735 g.
0.248	0.625	0.674	0.745	0.800
0.236	0.619	0.660	0.720	0.768

10 : 5 : 102.5	10 : 5 : 108.75	10 : 5 : 115.0	10 : 5 : 121.25	10 : 5 : 127.5
0.722 g.	0.832 g.	0.865 g.	0.818 g.	0.929 g.
0.747	0.778	0.802	0.834	0.930
0.735	0.810	0.834	0.824	0.930

Strain Y, 2

10 : 5 : 2.5	10 : 5 : 77.5	10 : 5 : 83.75	10 : 5 : 90.0	10 : 5 : 96.25
* 0.720 g.	0.661 g.	0.647 g.	0.743 g.	0.658 g.
0.511	0.720	0.750	0.692	0.804
0.511	0.691	0.699	0.718	0.731

* Accidental zinc culture.

10 : 5 : 102.5	10 : 5 : 108.75	10 : 5 : 115.0	10 : 5 : 121.25	10 : 5 : 127.5
0.787 g. 0.685	0.794 g. 0.819	0.863 g. 0.767	0.829 g. 0.854	0.887 g. 0.824
0.736	0.807	0.815	0.842	0.856

Fructification initiated in all cultures (except the accidental zinc) at about the same time—second day. Spores in all cultures brown, except that the membranes of all but the two magnesium sulphate concentrations (77.5 and 83.75) of the W strain had a ring of jet black spores on the margins.

16. The effect of increasing the concentration of the magnesium sulphate in the Pfeffer solution.

Strain W, 3		Strain Y, 3	
10 : 5 : 2.5	10 : 5 : 130	10 : 5 : 2.5	10 : 5 : 130
* 0.908 g. 0.127 0.078 0.081 0.133	0.899 g. 0.959 0.859 0.895 0.827	0.327 g. 0.362 0.341 0.206 0.268	0.756 g. 0.851 0.924 0.784 0.725
0.105	0.888	0.301	0.808

Complete sporulation took place in the W strain in 2½ days; in the Y strain in 2 days.

* Accidental zinc culture.

Here again (experiments 7 to 16) we note only a decrease in growth resultant upon decreasing the concentration of one or more of any of the three components of the Pfeffer nutrient solution. As a result of their increase we obtain, however, an increased growth. In the case of NH_4NO_3 and KH_2PO_3 the acceleration of growth is but a moderate one in spite of the fact that these compounds were added in so high amounts that the action of the impurities they doubtless contained might have been manifested. Only magnesium sulphate, it will be noted, gave results at all comparable to those with zinc, and then only in very high concentration. The high concentrations used renders it very probable that the action of the magnesium sulphate is to be attributed to the presence of zinc, etc., in traces. Since we have seen in previous experiments that the zinc optimum is approximately 0.1 mg. Zn/L, we can calculate that in a ten percent solution of magnesium sulphate zinc need only be present in 0.0001 percent impurity to be thus added in sufficient amount to cause maximum acceleration of growth. The results obtained with magnesium sulphate are discussed more in detail later. The evidence is quite conclusive, however, that neither the ammonium nitrate, the potassium phosphate, nor the magnesium sulphate is present in greater than optimum or in toxic concentration.

On inspection of the data (see table below) as to the ions whose presence results in an acceleration of the growth of *A. niger*, it is obvious that the majority are salts of heavy metals. The salts of the heavy metals, when the anion is that of a strong acid, hydrolyze in aqueous solution, resulting, as a rule, in increased acidity of the solution. Of the seventeen ions ($[H^+]$ is included) contained in this list, thirteen are reported as acting in this manner. The apparent exception in the case of the ferrous and manganous ions disappears on recognition of the ease with which their solutions are oxidized when in contact with the atmosphere. Ferric and manganic (14) ions behave similarly to the other heavy metal ions, as is generally reported.

In the case of only two of the ions listed above (F^- and $SiO^-{}_3{}^-$) does the hydrolysis of the majority of their salts lead to decreased acidity of the solution. In the case of the fluorides, the ammonium salt (NH_4F) from what can be gathered from data at our disposal, appears to hydrolyze in aqueous solution (14) with increased acidity due to the formation of HNH_4F_2.

A. niger

Compounds Reported as "Stimulants"

Compound	Max. Yield / Control	Optimum of "Stimulant"	Author	% Hydrolysis at 25° C. (27)	Solution Acid to Litmus (40)
$ZnSO_4$	4.69 /1.55	0.1 mgZn/L	Javillier (21)	m/5 = 0.0075	+
$MnSO_4$	0.982/0.610	10 g.Mn/L	B. & J. (4)	None?	o
$CdSO_4$	0.750/0.277	0.01 mgCd/L	Lepierre (29)	m/5 = 0.017	+(*)
$BeSO_4$	0.490/0.281	0.01 mgBe/L	" (30)		+(66)
$UO_2(NO_3)_2$			" (31)		+
$HgCl_2$	0.630/0.341	0.0013%	Ono (49)	m/256 = 1.43	+
$MnCl_4$	0.380/0.245	0.004%	Richards (57)		+
$CoSO_4$	0.805/0.550	0.002%	"	m/32 = 0.015	+(*)
$CoSO_4$	0.872/0.297	0.014%	Ono (49)		+
$NiSO_4$	0.785/0.200	0.033%	Richards (57)	m/32 = 0.044	+(13)
$NiSO_4$	0.404/0.262	0.014%	Ono (49)		+
$FeSO_4$	0.892/0.275	0.132%	Richards (57)	None?	o
$CuSO_4$	0.352/0.218	0.003%	Ono (49)	m/5 = 0.05	+
NaF	0.640/0.330	0.002%	Richards (57)		−(66)
NaF	0.325/0.199	0.0025%	Ono (49)		−
LiCl	0.410/0.280	0.016%	Richards (57)		?
Li_2SO_4	0.560/0.235	0.5%	"		?
$LiNO_3$	0.428/0.300	0.008%	"		+(*)
Na_2SiO_3	0.575/0.350	0.004%	"		−(66)
$Al_2(SO_4)_3$	0.260/0.205	0.002%	"		+
$Fe_2(SO_4)_3$	0.6+/0.1+		Steinberg		+
Acids	0.3 /0.13		"		+

* Tested the Kahlbaum compounds personally. In the case of the lithium salts only the nitrate was available.

I have repeated Richards' (57) experiments with sodium silicate. The results of my experiments are negative. They are tabulated below (experiments 17 and 18).

17. The effect of adding sodium silicate to the culture medium.

Strain W, 11

Control	Na_2SiO_3 (Kahlbaum)	
0.066 g.	0.002%	0.073 g.
0.035	0.008	0.073
0.086	0.098	0.078
0.084		
0.068		

There appeared no difference with respect to sporulation between the controls and the sodium silicate cultures. The acidities of all cultures at the time of harvest corresponded to $p_H = 3-4$.

18. The effect of adding sodium silicate to the culture medium.

Strain W, 11			
Na_2SiO_3 (Kahlb.)	Yield	Na_2Sio_3 (Kahlb.)	Yield
0.%	0.176 g.	0.12%	0.236 g.
0.002	0.195	0.18	0.139
0.01	0.154	0.26	0.126
0.05	0.230	0.32	0.144
0.08	0.207	0.40	0.162

No differences in spore formation appeared. Acidities at the time of harvest were equivalent to $p_H = 3 - 4$.

My cultures showed none of the ordinary "stimulation" effects, and I am inclined to associate these results with the fact that the addition of the silicate does not result in the increased acidity of the nutrient solution.

Further exceptions to this general rule that "stimulants" are electrolytes whose cations form weakly ionized bases of low solubility (*i.e.*, dissociate hydrolytically in the presence of an anion of a strong acid so as to cause an increase in acidity) are found possibly in experiments by Richards (57) and Yasuda (69) indicating that alkaloids, and by Miss Latham (28) indicating that chloroform, may cause growth "stimulation."

Finally there remain the substances used by Schulz (62) in his studies on acceleration of respiration with yeast: $HgCl_2$, I, KI, CrO_3, sodium salicylate, formic acid, and arsenious acid; and those used by Fred (16) in his studies on bacteria: ether, carbon disulphide, potassium dichromate, $CuSO_4$, and salvarsan. Of these thirteen substances, six agree in their ability to cause an increase in acidity; two must be grouped with chloroform as comparatively chemically inert, and the position of the others: I (and KI), Br, sodium salicylate, and salvarsan are doubtful.

It is to be noted for all these cases that practically the difficulty is not to induce the appearance of the phenomena associated with zinc "stimulation," but to prevent their occurrence. Substances exercising an action analogous to that of zinc may very easily be accidentally introduced through use of improper glassware (64) and impure chemicals. Particularly is one to exercise great caution in the interpretation of results obtained with compounds in high concentration, since any impurity they contain may thus be presented to the organism in effective concentration. An impurity of one-thousandth of one percent may cause the introduction in five percent solution of a compound of 0.5 mg./L impurity.

Further I have no wish to suggest that the degrees of growth "stimulation" are always the result of varying acidities. Without doubt, it is possible that even if the majority of these substances actually function through their effect on the acidity of the nutrient solution, others by influencing the organic processes at another stage, or in another way, may bring about the same result.

The effect of increased acidity, and of decreased acidity as well, of the Pfeffer nutrient solution are indicated in the following experiments. Increased acidity of the nutrient solution was brought about through the addition of different free concentrated acids immediately after sterilization. In order to obtain cultures of lower acidity than the Pfeffer solution, free alkali was added. In all cases full precautions were taken for the exclusion of zinc, etc.

19. The effect of increasing the acidity of the culture medium.

	Strain W, 5	
Control	H_3PO_4 (Kahlb. kryst.)	
0.133 g.	0.1 cc./flask	0.279 g.
0.124	0.2	0.302
0.146	0.3	0.307
0.140	0.4	0.232
0.121	0.5	0.178
0.133		

20. The effect of an increase or of a decrease in acidity of the culture medium.

	Strain W, 6	
Control	KOH (5:6)*	
0.107 g.	0.1 cc./flask	0.109 g.
0.126	0.2	0.055
0.126	0.3–0.5	No growth
0.120		

NH_4OH (Sp. Gr. 0.90)		HNO_3 (Sp. Gr. 1.42)	
0.1 cc./flask	0.065 g.	0.1 cc./flask	0.200 g.
0.2	0.030	0.2–0.5	No growth.
0.3	0.023		
0.4–0.5	No growth.		
HCl (Sp. Gr. 1.18)		H_2SO_4 (Sp. Gr. 1.84)	
0.1 cc./flask	0.291 g.	0.1–0.5 cc./flask	No growth.
0.2	0.129		
0.3	0.106		
0.4–0.5	No growth		

The hyphae in the cultures to which KOH or NH_4OH was added were watery and translucent in appearance. Sporulation in these cultures was very good. In those to which HNO_3 or HCl was added the hyphae were opaque, the membranes wrinkled, and spore formation was retarded.

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* Baker's "Analysed": NH_4OH, KOH, HNO_3, HCl, $Ba(OH)_2$, tartaric acid. Baker's "U.S.P.": acetic acid. Baker's "special": oxalic acid, H_2SO_4. Kahlbaum: H_3PO_4 kryst.

21. The effect of an increase or of a decrease in acidity of the culture medium.

Strain W, 7

Control	NH_4OH (Sp. Gr. 0.90)		KOH (5:6)	
*0.907	0.02 cc./flask	0.214 g.	0.02 cc./flask	0.141 g.
0.211	0.04	0.135	0.04	0.140
0.196	0.06	0.158	0.06	0.185
0.112	0.08	0.224	0.08	0.182
0.112	0.10	0.131	0.10	0.183
0.126				

HNO_3 (Sp. Gr. 1.42)		HCl (Sp. Gr. 1.18)		H_2SO_4 (Sp. Gr. 1.84)	
0.02 cc./flask	0.130 g.	0.02 cc./flask	0.112 g.	0.02 cc./flask	0.244 g.
0.04	0.233	0.04	0.115	0.04	0.336
0.06	0.282	0.06	0.211	0.06	0.239
0.08	0.289	0.08	0.329	0.08	0.110
0.10	0.266	0.10	0.274	0.10	0.028

* Accidentally used a Jena flask.

I accidentally added the NH_4OH, KOH, HNO_3, etc., to the flasks before sterilization. Spore formation in all except the cultures to which acids were added was excellent. Good sporulation in all cultures with HNO_3 and HCl. H_2SO_4 : 0.02 cc., excellent spore formation, translucent hyphae; 0.04 cc., opaque hyphae; 0.06 cc., dense white hyphae, slight retardation of spore formation; 0.08 cc., thin, dense white membrane, few spores; 0.10 cc., submerged hyphae, no membrane.

22. The effect of an increase in acidity of the culture medium.

Strain W, 7

Control	Tartaric Acid		Acetic Acid (99%)	
0.078 g.	0.5 cc./flask	0.184 g.	0.1 cc./flask	0.236 g.
0.082	1.0	0.305	0.2–0.5	No growth
0.091	1.5	0.352		
0.087	2.0	0.387		
0.090	2.5	0.407		
0.086				

A time retardation of spore formation was evident in cultures containing 2.0 and 2.5 g. tartaric acid to the 50 cc. of nutrient solution.

23. The effect of an increase or of a decrease in acidity of the culture medium.

Strain W, 8

Control	NH_4OH (Sp. Gr. 0.90)		KOH (5:6)	
0.131 g.	0.05 cc./flask	0.166 g.	0.02 cc./flask	0.107 g.
0.135	0.1	0.055	0.05	0.081
0.112	0.2	0.039	0.1	0.057
0.111	0.3	No growth	0.2	0.075
0.136	0.4	" "	0.3	No growth
0.125				

$Ba(OH)_2$ (0.084 N)		HNO_3 (Sp. Gr. 1.42)	
0.1 cc./flask	0.117 g.	0.02 cc./flask	0.070 g.
0.3	0.101	0.05	0.125
0.5	0.157	0.08	0.169
1.0	0.180	0.1	0.336
1.5	0.163	0.2	No growth

H_3PO_4 (Kahlb. Kryst.)

0.1 cc./flask	0.303 g.
0.2	0.286
0.3	*0.905
0.4	0.266
0.5	0.180

H_2SO_4 (Sp. Gr. 1.84)		HCl (Sp. Gr. 1.18)	
0.02 cc./flask	0.188 g.	0.05 cc./flask	0.107 g.
0.04	0.244	0.1	0.261
0.06	0.260	0.2	0.227
0.08	0.167	0.3	0.039
0.10	0.034	0.4	No growth

$HC_2H_3O_2$ (99%)

0.02 cc./flask	0.225 g.
0.05	0.253
0.08	0.279
0.1	0.280
0.2	No growth

$H_2C_2O_4$		+ 0.1 Mg. Zn/L	
0.1 g./flask	0.202 g.	0.08 cc. H_2SO_4/flask	0.572 g.
0.2	0.289		
0.3	0.337	0.02 cc. NH_4OH/flask	0.203
0.4	0.233		
0.5	0.043		

The reactions of the freshly prepared cultures were all acid to litmus except: KOH—0.2 and 0.3 cc.; and NH_4OH—0.2, 0.3, and 0.4 cc.

On the second day sporulation was complete in the controls: NH_4OH—0.05 and 0.1 cc.; $Ba(OH)_2$—all; $HC_2H_3O_2$—0.02, 0.05, and 0.08 cc. On the seventh day sporulation was completed in the controls, and in the cultures to which KOH, NH_4OH, $Ba(OH)_2$, and $HC_2H_3O_2$ had been added. The lowest concentrations of acid causing partial suppression of spore formation were: HNO_3—0.1 cc.; H_3PO_4—0.4 cc.; H_2SO_4—0.06 cc.; HCl—0.2 cc. and $H_2C_2O_4$—0.2 g.

As respects the cultures to which zinc had been added, those containing H_2SO_4 was practically sterile, those containing NH_4OH black with spores.

* Accidental zinc culture.

24. The effect of an increase or of a decrease in acidity of the culture medium.

Strain W, 11

Control	0.1 Mg. Zn/L	0.2 Cc. NH_4OH/Flask	0.2 Cc. H_3PO_4/Flask
0.066 g.	0.869 g.	0.016 g.	0.229 g.
0.035	0.853	———‡	0.233
0.086	0.862	0.026	0.226
0.084	0.782	0.024	0.262
† 0.677	0.911	———	* 0.902
0.068	0.855	0.022	0.238

Acidities on harvest corresponded to $p_H = 3–4$ (control, NH_4OH); 2–3 (H_3PO_4), and 1–2 (0.1 mg. Zn/L).

* Accidental zinc culture.

† Accidental stimulation resulting from addition of methyl violet 6 B extra Grübler, which may or may not be due to impurities.

‡ Not inoculated: p_H on harvest < 6.8; of the inoculated flasks $p_H = 3–4$.

The addition of acid to the Pfeffer nutrient solution resulted (experiments 19 to 24 inclusive) in an acceleration of growth. The maximum acceleration of growth, no matter which of the acids was used, was but slightly more than double that of the control. The effect of the addition of alkali to the Pfeffer solution was a diminution of the yield. Furthermore, the effect of the increased acidities of the cultures was to cause a retardation or suppression of spore formation as distinguished from the effects of decreased acidity which did not cause the exhibition of the same phenomenon.

All seven acids used in the above described experiments gave results essentially similar; an indication that these results are due to the hydrogen ion primarily and not to the anions. The acceleration of growth due to tartaric acid, however, needs to be verified, since I am inclined to attribute its action, at least in the sample used, to the presence of impurities. As a result of increased acidity, it will be noted, the yield can be doubled; while a decrease in the acidity of the Pfeffer solution acts in an opposite manner. My experiments serve, therefore, to confirm the long recognized fact that a fluctuation in the acidity brings about a corresponding variation in the yields; and moreover, that the acidity of the Pfeffer solution is sub-optimal for the growth of *A. niger*.

I have studied the appearance of the mycelia and of the modification of sporulation in cultures on Pfeffer solution of varying acidity. In all respects the agreement between the effects of increased acidity and the effects of ions like zinc and iron appears to be complete. The effects of decreased acidity and of increased acidity of the Pfeffer nutrient solution are distinct and opposite. Excessive acidity or alkalinity may, it is true, entirely prevent growth. Within these limits, however, an acidity lower than that of the Pfeffer solution ($p_H = 3–4$) results in a decreased yield, the hyphae are dark and watery in appearance—translucent—and the thin, flat membranes are black with spores. Increased acidity causes increased growth, the hyphae are white or light tan in color and heavy-walled—opaque—and the thickened and wrinkled membranes may show a retardation of spore formation.

The appearance and yields of the cultures in Pfeffer solution of higher acidity (greater than $p_H = 3–4$) are entirely similar to those containing zinc and iron. Cultures of lower acidity than that of the Pfeffer solution are similar to those from which zinc, etc., ions have been excluded. Moreover, a regular transition in the appearance and yields of the cultures is apparent from those of maximum alkalinity to those of maximum acidity.

The phenomena of chemical "stimulation" of *A. niger*: (*a*) increased growth, (*b*) increased respiration, (*c*) increased fermentative activity, (*d*) modification of the character of the cell walls, and (*e*) retardation or suppression of sporulation, are quite the same as those associated with the action of the hydrogen ion on the fungus.

Assuming that the H-ion concentration is a source of "stimulation," it should of necessity follow that a modification of the hydrogen-ion concentration of the Pfeffer nutrient solution would result in the modification of the optimum concentration of other "stimulants" for growth, and in such a sense that increased acidity brings about a decrease in the optimum of the "stimulant" and a decrease in the acidity an increase in the optimum of the "stimulant." Some evidence bearing on this question is furnished in the experiments of Richards (57), who has already pointed out that in the presence of the optimum iron concentration, the addition of zinc causes no further increase in growth. In the case of manganese and zinc, Bertrand and Javillier (5) found that even a further increase in yield took place in optimum concentration of either if the other was added. This modification of the optimum is easily demonstrated, as reference to the following experiments (25, 26) will show. See also experiment 23.

25. The effect of an increase or of a decrease in acidity of the culture medium on the growth accelerations obtained by addition of zinc.

Strain W, 8

——	+ 0.2 cc. NH_4OH	+ 0.2 cc. H_3PO_4
0.136 g.	0.015 g.	0.329 g.
0.200	0.020	0.313
0.139	0.025	0.334
0.135	0.018	0.318
0.095	0.024	0.299
0.141	0.020	0.319

Mg. Zn/L	——	+ 0.2 Cc. NH_4OH	+ 0.2 Cc. H_3PO_4
0.025 g.	0.358 g.	0.060 g.	0.614 g.
0.05	0.519	0.111	0.787
0.1	0.820	0.241	0.879
1.0	0.929	0.651	0.903
10.0	0.945	0.661	0.866
25.0	0.950	0.767	0.866
50.0	0.957	0.704	0.852
100.0	0.972	0.598	0.605

ACIDITIES OF THE CULTURE NUTRIENT SOLUTIONS AT TIME OF HARVEST

Mg. Zn/L	——	+ 4 Cc. NH_4OH/L	+ 4 Cc. H_3PO_4/L
—	p_H = 3–4	p_H = 3–4	p_H = 2–3
0.025	$\leqq 3$	$\leqq 3$	2–3
0.05	2–3	$\leqq 3$	2–3
0.1	1–2	2–3	2
1.0	1–2	2–3	1–2
10.0	1–2	2–3	1–2
25.0	1–2	2–3	1–2
50.0	1–2	$\leqq 3$	1–2
100.0	1–2	$\leqq 3$	1–2

The curves obtained by plotting yield against zinc concentration make somewhat more evident the variation of the zinc optimum as a result of the modification in acidity of the Pfeffer nutrient solution. Figure 1, including all the zinc concentrations used, is given for its general interest in depicting the types of curves obtained.

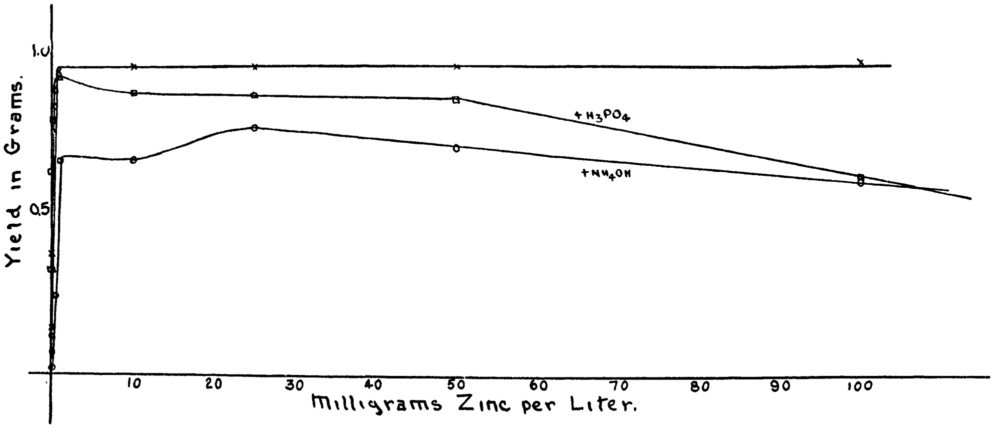

FIG. 1. For explanation see text.

Figure 2 shows more clearly the portions of these curves concerned with sub-optimal values.

We see that for any particular zinc concentration up to about 0.6 mg. Zn/L the amount of growth is least in the presence of NH_4OH and greatest in the presence of H_3PO_4. The zinc optimum also, as closely as these values permit of the location in each case, is about 0.06 mg. Zn/L in the presence of H_3PO_4; about 0.08 mg. Zn/L in the Pfeffer solution without any addition; while 0.1 mg. Zn/L may be taken as the optimum in the presence of NH_4OH. The value 0.08 mg. Zn/L for the nutrient solution

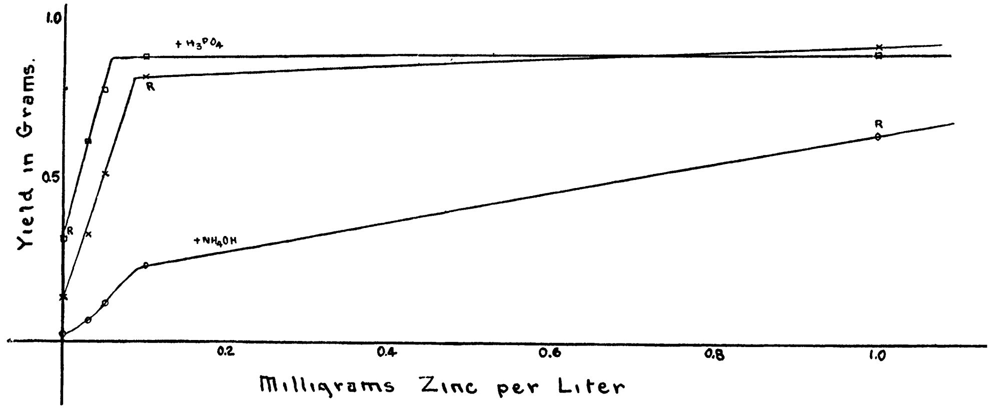

FIG. 2. For explanation see text.

without addition of either phosphoric acid or ammonium hydroxide compares well with the value (0.09 mg. Zn/L) found in another experiment (65). The curve obtained in the presence of NH_4OH is rather peculiar, and it may well be that the optimum should be taken in this case as in the neighborhood of 25 mg. Zn/L.

Increased acidity and presence of zinc ions exercise the same effect on the growth of *A. niger*—a positive acceleration. The more zinc the lower the acidity (and vice versa) required for the production of the maximum yield. The data already given show this to be the case. I am inclined to infer, although the authors do not themselves suggest this interpretation, that the same relation appears to hold for the action of iron and increased acidity on the rice plant (Gile and Carrero, 18, p. 521, tables 13 and 14); and for the action of aluminum and increased acidity (barley and rye) studied by Hartwell and Pember (19).

Not only the yields but also the fructification and the acidity attained in the cultures are correlated with the increased growth. In Figure 2 I have indicated by the letter "R" the lowest zinc concentrations exercising a perceptible effect on the sporulation of the cultures. These values are also contained in the following table (part of experiment 25). The hydrogen ion exponent (p_H) of the solutions at the time of harvest has also been added (see below).

Spore Formation in Pfeffer Solution

Partially retarded	
+ 0.2 cc. NH_4OH/flask + 1 mg. Zn/L	$p_H = 2–3$
+ 0.1 mg. Zn/L	1–2
+ 0.2 cc. H_3PO_4/flask	2–3
Sterile, etc.	
+ 0.2 cc. NH_4OH/flask + 100 mg. Zn/L (vigorous)	$p_H \leqq 3$
+ 100 mg. Zn/L (sparse)	1–2
+ 0.2 cc. H_3PO_4/flask + 1.0 mg. Zn/L (sterile)	1–2

Sporulation is therefore retarded through increased acidity and within limits favored by a decreased acidity. The first indication of retardation of spore formation occurs at an acidity corresponding to a hydrogen exponent of 2–3. In the ammonium hydroxide series spore formation was vigorous even in the presence of 100 mg. Zn/L. Here the low acidity ($p_H \overset{=}{<} 3$) is without doubt the correct explanation. The extent of the retardation of sporulation depends apparently on the acidity attained in the culture through the growth of the organism.

Use was made of the indicator method as given by Michaelis (41) for the estimation of the above listed acidities. The following results (part of experiment 25) were also obtained in this manner.

It will be noted that the cultures as a result of the growth of the organism attain an increased acidity in all cases, except in the Pfeffer solution without any additions and apparently in the Pfeffer solution to which phosphoric acid was added.

Pfeffer Nutrient Solution	Before Inoculation	At Time of Harvest
—	p_H = 3–4	p^H = 3–4
+ 0.1 mg. Zn/L	3–4	1–2
+ 1.0 "	3–4	1–2
+ 10.0 "	3–4	1–2
+ 0.2 cc. NH_4OH/flask	< 6–8	3–4
+ 0.2 cc. H_3PO_4/flask	2–3	2–3

Additional evidence of the increased acidity attained through the growth of the organism and its relation to the zinc content of the cultures is given in experiment 25 (see above). See also experiment 6, etc.

These acidity values though quite crude serve nevertheless to indicate clearly the existence of a parallelism between increase in growth, retardation of fructification, and increase in acidity of the medium. The maximum acidity attained is $p_H = 1–2$ and corresponds (though see H_3PO_4 series) to maximum growth. "Stimulation" due to iron salts (ferric and ferrous phosphate and ferric sulphate) results in the same acidity. This value, it is to be seen, is in agreement with that of Clark and Lubs (10) — $p_H = 1.7$, and that of Currie (11)—$p_H = 1.46$.

A few values obtained with the "Y" strain (experiment 26) are of essentially the same significance as those with the "W" strain (experiment 25).

26. The effect of an increase or of a decrease in acidity of the culture medium on the growth accelerations obtained by addition of zinc.

Strain Y, 8

Control	Yield	p_H on Harvest	Fructification
+ 0.2 cc. NH_4OH/flask	0.233 g.	2–3	Black with spores
+ 0.2 cc. H_3PO_4/flask	0.070	≦ 3	Retarded
+ 0.2 cc. NH_4OH/flask + 0.1 mg. Zn/L	0.420	2–3	Sterile
" " " + 1.0 " "	0.647	≦ 3	"
" " " + 10.0 " "	0.651	2–3	"
	0.595	2–3	"

I append also an experiment (experiment 27) in which acid variation was estimated by titration. Ten cubic centimeters of each culture was titrated with sodium hydroxide in the presence of methyl orange.

27. The effect of the addition of zinc to the culture medium on the rate of increase and the total acidities developed in the cultures.

Strain W, 11

Harvested	Yields		N/10 NaOH	
	No Zinc	0.15 Mg. Zn/L	No Zinc	0.15 Mg. Zn/L
1 day	0.084 g.	0.096 g.	0 cc.	0.53 cc.
2 days	0.117	0.297	0.86	2.80
3 "	0.088	0.507	2.45	5.25
4 "	0.149	0.670	1.23	5.95
5 "	0.230	0.781	2.10	7.00
7 "	0.176	0.910	1.93	7.88

Here we note that the total free acid in the culture solutions shows a regular and marked increase in total acidity only in the case of the zinc cultures.

It is of course always to be remembered that in order to get an acidity sufficient to be of even a barely perceptible physiological significance as a "stimulant," there must be a concentration of the salt of the heavy metal which is very high from the standpoint of its direct physiologically "stimulative effect." This is of course a strong argument against the assumption that the effect produced by "stimulants" is due to the increased acidity of the solution resulting from their hydrolytic dissociation. Nevertheless, I am inclined, on the basis of the very positive evidence that an increase in the acidity of the Pfeffer solution results in a marked "stimulation" of growth, to ascribe an important physiological effect to the increased H-ion concentration resulting directly from the dissociation of these salts in the culture medium. It is perhaps not impossible that in case the "stimulants" are adsorbed we may get a membrane concentration which would bring the acidity more nearly within the range of strengths necessary to produce directly "stimulative effects" on growth.

Thus it has been shown by Denham (13) that the hydrolysis of salts of this type can result in hydrogen-ion concentrations of considerable magnitude. It will suffice to take the values found in n/16 solution. This concentration is of course much greater than that often employed in the cultures.

Salt(n/16)	$[H^+]$	P_H(calc.)
Tl_2SO_4	0.609×10^{-2}	2.215
$CrCl_3$	0.234×10^{-2}	2.631
Al_2Cl_6	0.152×10^{-2}	2.818
$Al_2(SO_4)_3$	0.145×10^{-2}	2.839
$C_6H_5NH_3HCl$	0.114×10^{-2}	2.944
$NiSO_4$	0.440×10^{-4}	4.357
$CoSO_4$	0.107×10^{-4}	4.971
NH_4Cl	0.427×10^{-6}	6.370

The number of salts experimented with in this connection is so few that it is unfortunately impossible to ascertain whether any relation exists between p_H and the optima for, or the magnitude of the acceleration of, growth. Nevertheless, it is evident that their action does not depend entirely on the acidity attained through hydrolysis, since trivalent aluminum has comparatively little "stimulative effect" in comparison with bivalent cobalt and nickel. Further, the increased yield due to increased acidity is much less than that brought about by the addition of zinc and iron. The addition of the majority results, however, in growth accelerations of the same magnitude as that caused by increased acidity. This variation in effectiveness of the different "stimulants" from the point of view of increased acidity may be accounted for on the basis of impurities, valence, differences in ease of adsorption, degree of hydrolytic dissociation, or by the existence of a distinct action of the "stimulative" ion apart from its effecting an increase in acidity.

Another aspect of the difference in growth acceleration attained by increased acidity and by addition of the zinc ion lies in the probability that there is a variation in optimum acidity for *A. niger* at different stages of development (9). The growth in the Pfeffer solution ($p_H = 3-4$), I have repeatedly noticed, is greater than in the same solution to which phosphoric acid is added (0.2 cc.) for the first twenty-four hours. Nevertheless, the growth is as one to two at the end of seven days. The initial retardation is more than compensated for later. Experiments were therefore performed with the idea of ascertaining if a still greater growth acceleration with the hydrogen ion could be obtained through the gradual addition of free acid from day to day in comparison with that previously obtained by adding acid only before inoculation (experiments 28, 29, 30, 31).

All the culture flasks in experiments 28, 29, 30, and 31 were provided with a short glass tube inserted through the cotton plug of the flask so that the lower end almost reached to the bottom of the flask, while the upper end (plugged with cotton) projected above the cotton plug of the flaks.

28. The effect of a daily increase in acidity of the culture medium on the growth.

H_3PO_4/Flask				Strain W, 11
Initial	1 Day	2 Days	Total	Yield
0 cc.	0 cc.	0 cc.	0 cc.	0.156 g.
0	0.1	0.1	0.2	0.460
0.05	0.1	0.1	0.25	0.558
0.1	0.1	0.1	0.3	0.318

29. The effect of a daily increase in acidity of the culture medium on the growth.

Strain W, 11						
H_3PO_4/Flask						
Initial	First Day	2	3	4	Total	Yield
0 cc.	0 cc.	0 cc.	0 cc.	0 cc.	0 cc.	0.203 g.
0.2	0	0	0	0	0.2	0.374
0.3	0	0	0	0	0.3	0.443
0.4	0	0	0	0	0.4	0.400
0	0.05	0.1	0.1	0	0.25	0.328
0	0.05	0.1	0.1	0.1	0.35	0.564
0.05	0.1	0.1	0	0	0.25	0.398
0.05	0.1	0.1	0.1	0	0.35	0.371
0.1	0.1	0.1	0	0	0.3	0.520
0.1	0.1	0.1	0.1	0	0.4	0.504

+ 0.15 mg. Zn/L 0.910 g.

30. The comparative yield resulting from the addition of varying amounts of acid before inoculation and on the first day after inoculation.

Strain W, 11

H_3PO_4/Flask		H_3PO_4/Flask	
Added Before Inoculation	Yield	1 Day	Yield
0 cc.	0.204 g.	0.1 cc.	0.332 g.
0.2	0.327	0.2	0.267
0.3	0.638	0.3	0.435
0.4	0.544	0.4	0.464
0.5	0.246	0.5	0.285
0.6	0.166	0.6	0.202
0.7	0.049	0.7	0.215
0.8	0.040	0.8	0.186

H_3PO_4/Flask			
Initial	1st Day	2	Yield
0.1 cc.	0.3 cc.	0.1 cc.	0.232 g.
0.1	0.3	0.1	0.302
0.1	0.2	0.2	0.199
0.1	0.2	0.2	0.351

0.2 mg. Zn/L 0.985 g

31. The comparative yield resulting from the addition of varying amounts of acid before inoculation and on the first, second, and third days after inoculation.

Strain W, 11

H_3PO_4/Flask Added Before Inoculation		H_3/PO_4Flask Added 24 Hrs. After Inoculation		H_3PO_4/Flask Added 48 Hrs. After Inoculation	
0 cc.	0.183 g.	0.2 cc.	0.290 g.	0.2 cc.	0.241 g.
0.2	0.402	0.3	0.323	0.3	0.300
0.3	0.377	0.4	0.257	0.4	0.299
0.4	0.347	0.5	0.265	0.5	0.279
0.5	0.182	0.6	0.255	0.6	0.247
0.6	0.078				

H_3PO_4/Flask Added 72 Hrs. After Inoculation		Mg. Zn/L	Yield
0.2 cc.	0.244 g.	0	0.123 g.
0.3	0.325	0.025	0.339
0.4	0.274	0.05	0.378
0.5	0.310	0.075	0.447
0.6	0.215	0.1	0.540
		0.125	0.724
		0.15	0.755
		0.175	0.872
		0.2	0.909

The cultures of the zinc series were prepared as usual, *i.e.*, there was no tube penetrating through the cotton plug of the flask.

(*Concluded in the November number.*)